

Influence of *Escherichia coli* inclusion and soybean hulls based diets on ruminal biomethane and carbon dioxide productions in sheep



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ABSTRACT

In livestock production, ruminal fermentation leads to significant loss of digestible feed energy and increase methane (CH_4) and carbon dioxide (CO_2) productions. These gases are the major sources of greenhouse gases that cause environmental degradation and climate change. The present study aimed at investigating the sustainable control of CH_4 and CO_2 production from ruminal fermentation by evaluating ruminal inclusion of *Escherichia coli* (*E. coli*) on diets containing different levels of soybean hulls (SH) replacing corn grains (CG). Three different levels of mixed ration were prepared; CG was replaced with SH at three different levels (per kg dry matter (DM)): 0 g (control), 75 g (SH 75), and 150 g (SH 150). The *E. coli* was used at four doses: 0, 10, 20 and 40 $\mu\text{L/g}$ DM of substrate. The SH rations had decreased linear and quadratic ($P < 0.05$) effects on asymptotic gas production (GP). Interactions occurred between SH ration and *E. coli* doses ($P < 0.05$) on the fractional rate of GP. *E. coli* at all doses did not produce any effect on the CH_4 production parameters. However, the control had the highest CH_4 production at 40 $\mu\text{L/g}$ DM *E. coli* addition compared to other SH rations and their respective *E. coli* doses. SH ration linearly ($P = 0.006$) decreased asymptotic CO_2 production. The study established that SH ration and *E. coli* doses had no effect on the CH_4 production; however, they had a decreased effect on asymptotic GP. This study demonstrated that inclusion of SH 150 ration at different *E. coli* doses reduced asymptotic CO_2 production without effect on CH_4 production and this may be useful for the sustainable mitigation of CO_2 production from livestock production.

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1. Introduction

Globally, agricultural waste products are nutrient-rich and can serve as alternative sources of dietary feed for livestock production (Ahmed et al., 2015). In many developing countries, these waste products are burnt in the field thereby leading to pollution, climate change and environmental degradation (Kholif et al., 2014). In this way, agro-based industries contribute significant amounts of greenhouse gases (GHGs) such as carbon dioxide (CO_2) and methane (CH_4) to the atmosphere (Audsley and Wilkinson, 2014). Many researchers (Garnett et al., 2013; Gerber et al., 2013; Herrero et al., 2014) have reported the importance of sustainable intensification in agro-farming and highlighted the need to increase production efficiency and minimize the impact of waste products on

the environment.

Currently, agro-food based industries such as livestock production is among the leading contributors of the anthropogenic source of GHGs- CH_4 and CO_2 . Slade et al. (2016) in their recent investigation observed that two-third of the direct emissions is due to livestock production. According to the report of the Food and Agriculture Organization (FAO, 2006), animal production is responsible for 18% CH_4 and 9% CO_2 productions of all GHG emissions. Methane has a greater global warming effect (about 23 times) more than CO_2 (Rira et al., 2015) and accounts for 50–60% emitted GHG during ruminant production (Mirzaei-Aghsaghali et al., 2012). Methane production is also responsible for a net loss of 2–12% of total energy of feed in ruminant production (Mirzaei-Aghsaghali et al., 2012; Hristov et al., 2015). In the last decade, animal nutritionists and microbiologists have developed a keen interest in the manipulation of ruminal microbial ecology and its enteric fermentation kinetics. The basic aim of these manipulations is to

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improve animal feed utilization, enhance digestibility of fibrous feeds, reduce protein degradability, increase animal performance, minimize loss of dietary energy during rumen fermentation and also reduce CH₄ and CO₂ productions for eco-friendly animal production (Patra et al., 2006; Benchaar et al., 2007).

The use of agro-based waste products as unconventional feedstuff can be a major breakthrough in livestock production. Not only that they are cheap source of animal feeds, they are available all year round especially during dry season. Thus, they can guarantee continuous supply of animal feeds. They may also help to reduce CH₄ and CO₂ productions from livestock because ruminants depend on diet degradability and chemical composition of their feeds (Hristov et al., 2013; Elghandour et al., 2016a).

Soybean hulls have been used widely as a viable alternative and economic substitute in the ruminant (Costa et al., 2012) and horse (Esquivel-Velázquez et al., 2016) diets. However, due to low energy density and fibrous content of SH, their addition in ruminant diet requires inclusion of high energy feed ingredients such as corn grains (CG) and organic acid salts (Elghandour et al., 2016a). Castillo et al. (2004) reported that the addition of organic acid salts can stimulate propionic acid production in the rumen and reduce CH₄ emission by serving as a hydrogen (H₂) sink. Newbold et al. (2005) also reported that organic acid salts decreased CH₄ production by 8–7%. Elghandour et al. (2016a) investigated the ruminal CH₄ and CO₂ productions of SH by applying organic acid salts as a supplement and observed sustainable mitigation of the gases during livestock production. They also suggested that SH is not only useful as feedstuff for the production of ruminants but can reduce the environmental pollution caused by ruminal gases. However, they noted that supplementing organic acid salts with SH did not influence ruminal gas production but decreased CO₂ production.

In ruminants production, sodium or calcium propionate (Ferraro et al., 2009), organic salts (Elghandour et al., 2017), plant extract (Jiménez-Peralta et al., 2011; Salem et al., 2014a), *Saccharomyces cerevisiae* (Rodriguez et al., 2015) fibrolytic enzymes (Morsy et al., 2016), have been successfully used as rumen modifier. Inclusion of these ingredients as a ration for ruminants diets have shown positive effect on forage quality (Kholif et al., 2017a; b), feed utilization, digestibility, rumen fermentation, and animal performance (Valdes et al., 2015). Recently, it has been hypothesized that ruminal contamination with *E. coli* can affect ruminal microflora and ruminal fermentation thus influence GP, CH₄ and CO₂ productions (Elghandour et al., 2018). This may be due to antagonistic role *E. coli* plays on normal ruminal microflora which often leads to decreased fermentation of the ruminant diets and cause reduced CH₄ and CO₂ productions. Elghandour et al. (2018) reported that inclusion of prickly pear cactus rations with different *E. coli* doses reduced CH₄ and CO₂ productions in ruminants.

This present study aimed to evaluate the level of ruminal inclusion of *E. coli* on the nutritive value and greenhouse gas production of diets containing different levels of soybean hulls replacing corn grains. The reason for using *E. coli* doses with three different SH rations is to establish the optimal precision of the mixture of SH rations and *E. coli* doses that may work best in mitigation of CH₄ and CO₂ productions.

2. Materials and methods

2.1. Substrates and treatments

In this experiment, three different ration mixtures were prepared. In the mixtures, CG was replaced with SH at three different levels per kg dry matter (DM) namely: 0 g (Control), 75 g (SH 75) or 150 g (SH 150). Table 1 shows the ingredients and chemical composition of the experimental diets. *E. coli* was cultivated at the

Table 1
Ingredients and composition of the experimental diets^a.

	Control	SH 75	SH 150
Ingredients (g/kg DM)			
Oats straw	249	248	248
Steam rolled corn	250	175	100
Steam rolled barley	250	250	250
Wheat bran	120	110	120
Corn gluten feed	30	30	30
Soybean meal	30	30	20
Soybean hulls	0	75	150
Molasses	70	80	80
Vitamins/Minerals ^b	1.0	2.0	2.0
Chemical composition (g/kg DM)			
Organic matter	964	940	957
Crude protein	130	119	113
Neutral detergent fiber	356	428	340
Acid detergent fiber	121	130	122
Ether extract	24	22	23
Non-structural carbohydrates	455	371	481

^a Adapted from Esquivel-Velázquez et al. (2016).

^b Contained per kilogram: Vitamin A (12 000 000 IU), Vitamin D₃ (2 500 000 IU), Vitamin E (15 000 IU), Vitamin K (2.0 g), Vitamin B₁ (2.25 g), Vitamin B₂ (7.5 g), Vitamin B₆ (3.5 g), Vitamin B₁₂ (20 mg), Pantotenic acid (12.5 g), Folic acid (1.5 g), Biotin (125 mg), Niacin (45 g), Fe (50 g), Zn (50 g), Mn (110 g), Cu (12 g), I (0.30 g), Se (200 mg), Co (0.20 g).

concentration of 1×10^{10} in the laboratory of bacteriology of the Faculty of Veterinary Medicine and Animal Science, Autonomous University of the State of Mexico. The *E. coli* was used at four doses: 0, 10, 20 and 40 µL/g DM of substrate. The treatments were as follows: SH 75 treatment (at 0, 10, 20 and 40 µL/g DM *E. coli* doses) and SH 150 treatment (at 0, 10, 20 and 40 µL/g DM *E. coli* doses). The treatments were tested against the control treatment (without SH).

2.2. In vitro fermentation

Rumen inoculum was collected from two adult sheep (50 ± 2.5 kg body weight (BW)) fitted with a permanent rumen cannula. These sheep were fed *ad libitum* with a mixed ration of a concentrated commercial formula (PURINA®, Toluca, Mexico) and alfalfa hay in the ratio of 1:1 DM according to National Research Council (NRC, 2001). The collected rumen contents were flushed with CO₂, mixed and filtered through four layers of cheesecloth into a flask with O₂-free headspace. Each SH ration was weighed directly into a 120 mL serum bottles followed by addition of appropriate extract dose per gram DM *E. coli*. Consequently, exactly 10 mL of particle free rumen fluid was included to each of the serum bottles followed by 40 mL of the buffer solution of Goering and Van Soest (1970), without including trypticase.

Three incubation processes were conducted in three weeks. The rumen fluid bottles (as blanks) and the substrates bottles were incubated for 72 h. The rumen fluid bottles (as blanks) and the substrate bottles were incubated for 72 h at 39 °C. The volume of GP were measured every 2 h and after 24 h, two more measurements were taken at 48 h and 72 h with the help of Pressure Transducer Technique (Extech instruments, Waltham, USA) of Theodorou et al. (1994). The CH₄ and CO₂ productions were measured every 4 h and after 24 h two more measurements were taken at 48 and 72 h of incubation using Gas-Pro detector (Gas Analyzer CROWCON Model Tetra 3, Abingdon, UK). The *in vitro* incubation process is summarized in Fig. 1.

At the end of incubation, the fermentation process was stopped by swirling the bottles in ice. The bottles were then uncapped and the pH was determined using a pH meter (Conductronic pH 15, Puebla, Mexico). The contents of each bottle were filtered under vacuum through glass crucibles (coarse porosity no. 1, pore size

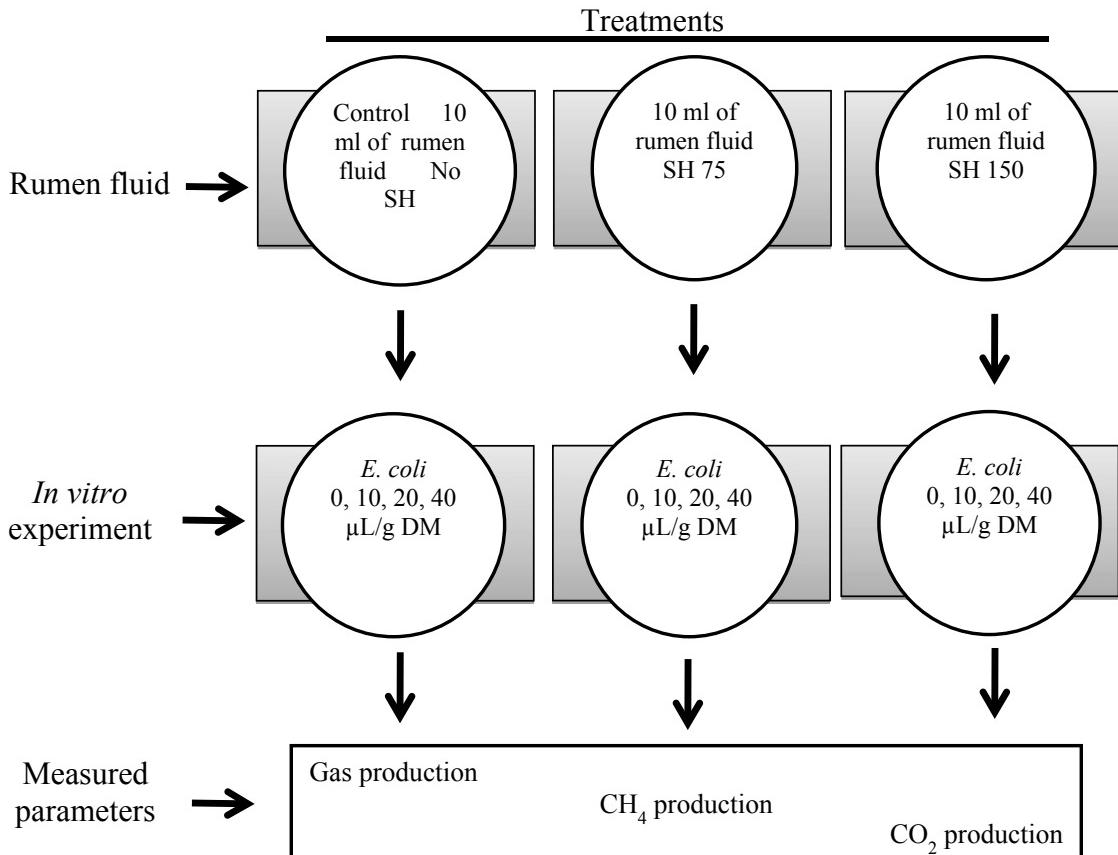


Fig. 1. Flowchart of the *in vitro* incubation process.

100–160 µm; Pyrex, Stone, UK) with a sintered filter to obtain the non-fermented residue for determination of degraded substrate after drying at 65 °C overnight.

2.3. Chemical analyses and calculations

Samples of the rations were analyzed for dry matter (DM) (method ID 934.01), ash (method ID 942.05), Nitrogen (method ID 954.01) and ether extract (method ID 920.39) using Association of Official Analytical Chemists (AOAC, 1997) official methods, Neutral detergent fiber (NDF) analyzed using the method of Van Soest et al. (1991) while acid detergent fiber (ADF) and lignin (AOAC, 1997; method ID 973.18) analyses were carried out using an ANKOM²⁰⁰ Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA) with the use of an alpha amylase and sodium sulfite.

Volumes (mL/g DM) of GP, CH₄ and CO₂ were used to estimate the fermentation kinetics parameters using the nonlinear model procedure of Statistical Analysis System (SAS, 2002) according to France et al. (2000) model as:

$$y = b \times [1 - e^{-c(t-Lag)}] \quad (1)$$

where, y is the volume (mL/g DM) of GP, CH₄ or CO₂ at time t (h); b is the asymptotic GP, the asymptotic CH₄ or the asymptotic CO₂ (mL/g DM); c is the fractional rate of fermentation (1/h), and Lag (h) is the discrete lag time prior to when any gas, CH₄ or CO₂ is released.

2.4. Statistical analyses

Data of each of the three runs within the same sample were

averaged prior to statistical analysis. Mean values of each individual sample were used as the experimental unit. Results of *in vitro* GP and rumen fermentation parameters were analyzed as a factorial experiment using the procedure of General Linear Model option of SAS (2002) as:

$$Y_{ijk} = \mu + R_i + D_j + (R \times D)_{ij} + E_{ijk} \quad (2)$$

where: Y_{ijk} is every observation of the i th ration type (R_i) with j th extract dose (D_j); μ is the general mean; $(R \times D)_{ij}$ is the interaction between ration type and extract dose; E_{ijk} is the experimental error. Linear, quadratic and cubic polynomial contrasts were used to examine response of different rations on increasing addition doses per gram DM *E. coli*. Statistical significance was declared at the level of $P < 0.05$.

3. Results

3.1. Gas production and fermentation

The levels of SH ration had decreased linear ($P = 0.001$) and quadratic ($P = 0.005$) effects on asymptotic GP. *E. coli* doses had no linear, quadratic and cubic effects on asymptotic GP. No ration and dose interaction were observed on asymptotic GP. However, *E. coli* doses had linear ($P < 0.001$) and cubic ($P = 0.003$) effects on the rate of GP. Inclusion of SH ration and *E. coli* doses had dependent interaction ($P < 0.05$) on the fractional rate of GP. Rational type linearly ($P = 0.006$) and quadratically ($P = 0.013$) affected the initial delay before GP began (Table 2, Fig. 2a–c).

The addition of SH ration had linear and quadratic effects

Table 2*In vitro* rumen gas, CH₄ and CO₂ kinetics of three different levels of soybean hulls (SH) as influenced by different doses of *E. coli* ($\mu\text{L/g DM}$).

Ration ^a	<i>E. coli</i>	pH and degradability		Gas production ^c			CH ₄ production ^d			CO ₂ production ^e		
		pH	DMD ^b	b	c	Lag	b	c	Lag	b	c	Lag
Control	0	5.68	543.2	373.1	0.078	1.11	34.0	0.029	9.91	149.2	0.029	4.08
	10	5.71	548.1	316.4	0.101	1.06	55.1	0.050	13.50	108.0	0.047	3.90
	20	6.04	572.0	366.3	0.093	1.06	50.0	0.032	10.70	219.0	0.052	4.14
	40	6.40	558.0	395.0	0.121	1.17	85.2	0.021	5.91	191.1	0.056	4.11
	SH75	0	6.46	498.3	268.1	0.075	1.40	35.1	0.028	14.30	118.2	0.045
SH150	10	6.40	537.4	229.3	0.092	1.62	19.0	0.043	21.20	135.3	0.034	3.33
	20	6.47	536.2	288.0	0.079	1.50	34.0	0.019	7.82	129.2	0.018	2.44
	40	6.49	497.1	230.2	0.122	1.30	22.2	0.113	10.30	97.4	0.040	3.51
	0	6.65	507.4	305.1	0.085	1.03	45.3	0.027	15.30	165.0	0.035	3.39
	10	6.59	530.0	235.3	0.215	1.66	54.4	0.033	9.11	92.3	0.074	4.78
Pooled SEM ^f	20	5.98	524.3	221.1	0.092	1.64	55.3	0.032	12.10	98.0	0.052	3.62
	40	6.10	529.0	230.0	0.252	1.37	38.0	0.036	8.53	81.1	0.061	4.08
	Ration effect	0.212	15.90	27.40	0.0191	0.118	16.70	0.015	4.814	24.41	0.014	0.784
	Linear	0.020	0.007	<0.001	<0.001	0.006	0.523	0.925	0.719	0.006	0.349	0.874
	Quadratic	0.024	0.032	0.005	0.003	0.013	0.036	0.072	0.363	0.257	0.079	0.212
<i>E. coli</i> effect	Linear	0.677	0.525	0.489	<0.001	0.691	0.458	0.057	0.140	0.619	0.304	0.881
	Quadratic	0.415	0.039	0.201	0.333	0.012	0.784	0.421	0.853	0.954	0.857	0.534
	Cubic	0.725	0.834	0.055	0.003	0.211	0.984	0.138	0.421	0.058	0.196	0.436
	Ration × Dose	0.058	0.689	0.320	0.005	0.063	0.507	0.039	0.699	0.070	0.518	0.742

^a SH 75, soybean hulls were included at 75 g/kg DM of total mixed ration; SH 150, soybean hulls were included at 150 g/kg DM of total mixed ration.^b DMD is the dry matter degradability (g degraded/g incubated).^c b is the asymptotic gas production (mL/g DM); c is the rate of gas production (h^{-1}); Lag is the initial delay before gas production begins (h).^d b is the asymptotic methane production (mL/g DM); c is the rate of methane production (h^{-1}); L is the initial delay before methane production begins (h).^e b is the asymptotic carbon dioxide production (mL/g DM); c is the rate of carbon dioxide production (h^{-1}); L is the initial delay before carbon dioxide production begins (h).^f SEM standard error of the mean.

($P=0.02$) on pH but *E. coli* inclusion at different doses produced no effects on pH. The control had the highest dry matter degradability (DMD) of 572 mg/g DM at 40 $\mu\text{L/g DM}$ *E. coli* while the lowest DMD of 497 mg/g DM was recorded at SH 75 ration. The SH ration had decreased linear and quadratic effects ($P<0.05$) on DMD. *E. coli* showed a quadratic effect ($P<0.05$) on DMD, however no ration and dose dependent effects were observed on DMD (Table 2).

3.2. CH₄ and CO₂ productions

The SH ration had quadratic effects ($P=0.04$) on asymptotic CH₄ production. *E. coli* at different doses had no linear, quadratic or cubic effects on asymptotic CH₄ production. Interactions between SH ration and *E. coli* doses were observed ($P<0.04$). No linear or quadratic effects were observed with the inclusion of SH ration and *E. coli* doses on the rate or initial delay before CH₄ productions. The SH ration had quadratic effects ($P<0.05$) for 24 h and 48 h incubation time on mL/g incubated DM and mL/g degraded DM of CH₄ production (Fig. 3a–c). The SH ration had linear effects at 24 h ($P=0.018$) and 48 h ($P=0.027$) of incubation during proportional CH₄ production. SH ration and *E. coli* dose interaction occurred at 8 h incubation ($P=0.010$) (Table 3).

The SH ration produced linear effects ($P=0.006$) on asymptotic CO₂ production. The control had the highest asymptotic CO₂ production of 219 mL/g DM at 20 $\mu\text{L/g DM}$ *E. coli* inclusion while SH 150 had the lowest asymptotic CO₂ production of 81 mL/g DM at 40 $\mu\text{L/g DM}$ *E. coli* inclusion. Addition of SH ration had no linear or quadratic effects on the rate of CO₂ production. No effect of SH rations and *E. coli* doses interactions was observed on the rate of CO₂ production and initial delay before CO₂ production began. However, in the CO₂ production of mL/g incubated DM, linear and quadratic effects were observed ($P<0.05$) on the addition of SH rations at 8 h, 24 h and 48 h incubation time (Fig. 4a–c). The SH ration had quadratic effect ($P<0.05$) at all incubation time but had linear effects ($P<0.05$) at 24 and 48 h incubation time of mL/g degraded DM of

CO₂ production. No linear, quadratic or cubic effects were noted on the inclusion of different *E. coli* doses for mL/g incubated DM, mL/g degraded DM and proportional CO₂ production (Table 4).

4. Discussion

Worldwide, agricultural by-product is one of the major contributors of GHGs such as CH₄, CO₂, and NO₂, of which livestock production accounts for the two-thirds of the direct emissions (Slade et al., 2016). Enteric fermentation from ruminants digestive system produces CH₄ and CO₂ that contribute significantly to environmental degradation and pollution attributed to human activity. One of the greatest challenges that animal nutritionists are facing today is how to reduce greenhouse gas production in livestock farming for cleaner environmental production through feed formulation. Mitigating these gases (CH₄ and CO₂) from ruminant production will not only decrease greenhouse gases from agricultural by-products but also will significantly reduce the net feed energy loss and improve animal production (Eckard et al., 2010; Hernández et al., 2017). This present study used *in vitro* GP technique a simple, sensitive and powerful method to evaluate *in vitro* gas production, rumen fermentation kinetics (Salem et al., 2014a; b) and efficacy of feed utilization.

4.1. Gas production and fermentation

Generally, the level of digestibility and fermentation of ruminant feeds are measured by gas released in *in vitro* rumen fermentation kinetics. Higher GP indicates higher digestibility, better fermentability and increase in microbial protein production which result in better accessibility of nutrients to rumen microorganisms (Getachew et al., 2004). Inclusion of SH as a replacement for CG, linearly and quadratically decreased asymptotic GP, indicating decreased fermentability and digestibility of the diets (Fig. 2a–c). This finding is not in consonance with the report of

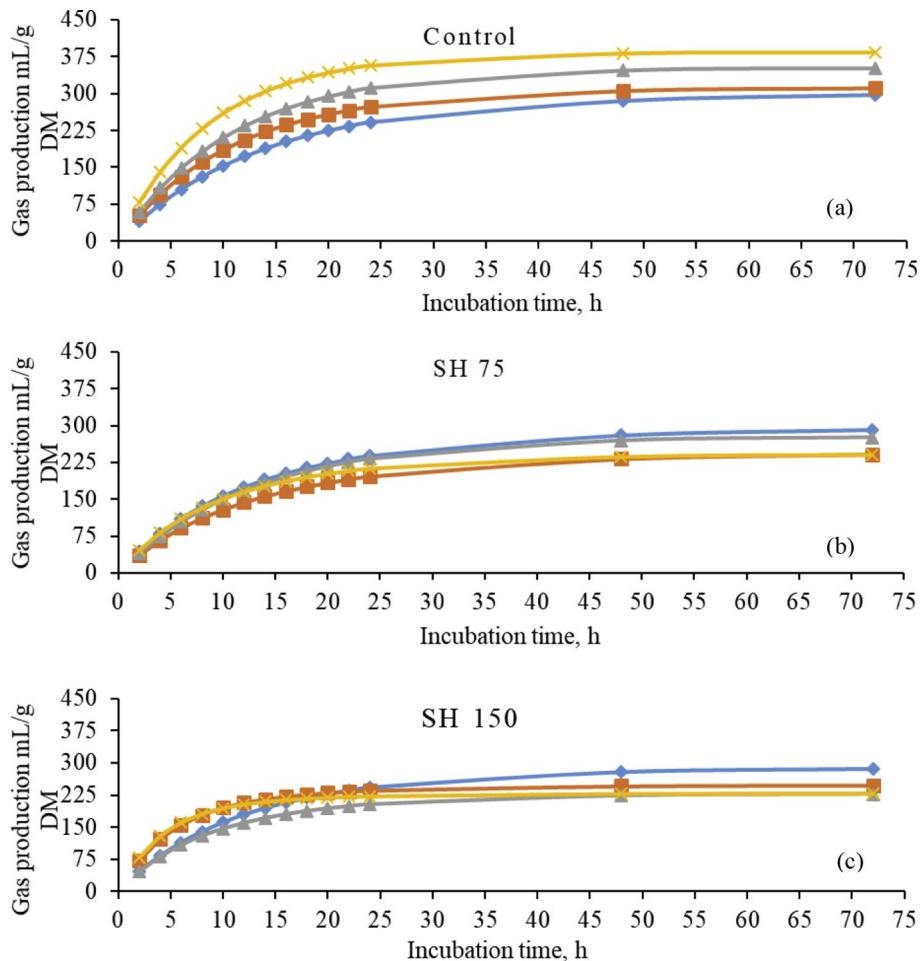


Fig. 2. a-c. *In vitro* rumen gas production (mL/g incubated DM) of three different levels of soybean hulls as affected by different levels of *E. coli* at 0 (-◆-), 10 (-■-), 20 (-▲-) and 40 (-×-) $\mu\text{L/g}$ DM. Control: corn grain was replaced by soybean hulls at 0 g/kg DM; SH 75: soybean hulls were included at 75 g/kg DM of total mixed ration; SH 150: soybean hulls were included at 150 g/kg DM of total mixed ration.

(Elghandour et al., 2015, 2016b), who observed significant increase in asymptotic GP with increasing level of prickly pear cactus flour replacement with corn grain. They reported that the observed increase in GP may be due to the availability of fermentable carbohydrate which has the tendency to promote microbial growth (Forsberg et al., 2000). Guerrero et al. (2012) opined that increase in GP improves availability of nitrogen and promote higher nutrients to rumen microbes. *E. coli* inclusion did not affect asymptotic GP. The inability of *E. coli* doses to positively influence the asymptotic GP suggests a decreased availability of carbohydrate for the microbial growth. This result agrees with the findings of Elghandour et al. (2016a) who reported that inclusion of organic acid salt on prickly pear cactus flour had no effect on the asymptotic GP. Ruiz et al. (2013) reported that inclusion of fibrolytic enzymes did not affect GP. This result suggests that replacement of SH with CG lack the propensity to improve ruminant feed intake and performance. The control rations had a lower lag phase when compared to the SH ration. This agrees with the report of (Ferraro et al., 2016) suggesting a faster adaptation of the microbes to the CG ration indicating its availability to provide more proportion of nutrient to the ruminant animals than the SH. However, the observed higher lag phase of 10 and 20 $\mu\text{L/g}$ DM *E. coli* doses indicate delay in microbial degradation and adaptation of the rumen microbes to the diets. Ferraro et al. (2016) reported that consistent feeding of sheep with diets supplemented with energy additives improves microbial

adaptability and reduces lag phase. This is in agreement with the report of Elghandour et al. (2016a) but in contrast to the findings of Rodriguez et al. (2015) that *Saccharomyces cerevisiae* reduced lag time. Elghandour et al. (2015) reported that fibrolytic enzymes have no influence on lag time of GP. Furthermore, the decrease in GP when SH replaced CG did not enhance nutrient availability to the rumen microbes. No ration and dose dependent interactions occurred indicating that there was no observable synergy between CG replacements with SH and *E. coli* doses on asymptotic GP.

4.2. CH_4 and CO_2 productions

The SH ration had quadratic effects on asymptotic CH_4 . *E. coli* at different doses had no effect on asymptotic CH_4 production as shown in Fig. 3a-c. No ration and dose dependent interactions occurred on asymptotic CH_4 production. However, the control had the highest asymptotic CH_4 production of 85 mL/g DM *E. coli* dose of 40 $\mu\text{L/g}$ DM. The control also had the highest mL/g incubated DM and mL/g degraded DM of CH_4 at the same *E. coli* dose. The least asymptotic CH_4 production, mL/g incubated DM and mL/g degraded DM was observed at the dose of 10 $\mu\text{L/g}$ DM of SH 75 ration. This result is similar to the findings of Elghandour et al. (2016a) who reported that replacing CG with SH did not affect CH_4 production. In their work, Elghandour et al. (2016a) reported that addition of organic acid salts doses had no effect on mitigation

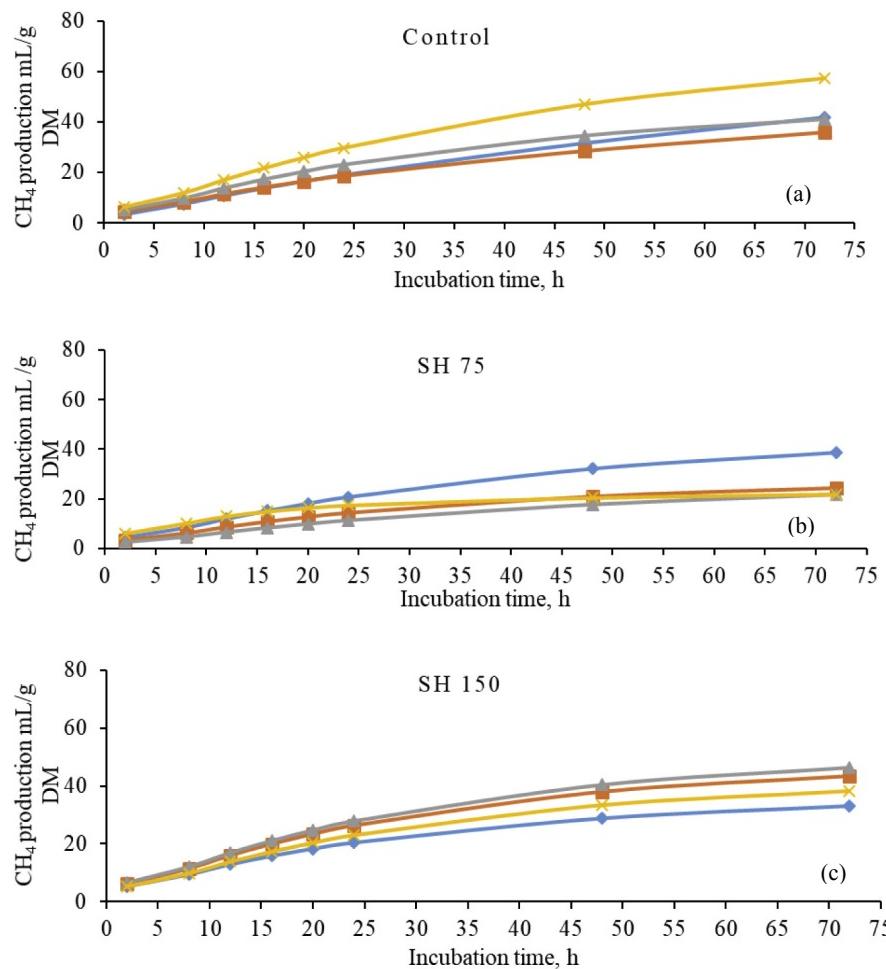


Fig. 3. a-c. *In vitro* CH₄ production (mL/g incubated DM) of three different levels of soybean hulls (SH) as influenced by different doses of *E. coli* at 0 (-◆-), 10 (-■-), 20 (-▲-) and 40 (-×-) µL/g DM. Control: corn grain was replaced with soybean hulls at 0 g/kg DM; SH 75: soybean hulls were included at 75 g/kg DM of total mixed ration; SH 150: soybean hulls were included at 150 g/kg DM of total mixed ration.

Table 3

Proportional *in vitro* CH₄ production as a percent of total gas production of three different levels of soybean hulls (SH) as influenced by different doses of *E. coli* (µL/g DM).

Ration ^a	<i>E. coli</i>	CH ₄ production						Proportional CH ₄ production		
		mL/g incubated DM			mL/g degraded DM					
		8 h	24 h	48 h	8 h	24 h	48 h	8 h	24 h	48 h
Control	0	7.23	16.50	23.29	13.60	30.66	43.27	3.99	5.09	6.35
	10	8.57	18.41	26.99	15.50	33.15	48.37	5.04	7.05	9.97
	20	10.60	25.35	37.55	18.79	44.87	66.39	5.63	7.62	9.97
	40	12.91	33.13	53.29	23.02	59.06	95.00	5.13	8.61	13.11
SH75	0	6.60	16.19	24.72	13.64	33.55	51.44	5.84	8.09	10.92
	10	5.13	11.14	15.23	9.60	20.85	28.53	5.32	5.82	6.40
	20	3.70	9.62	15.92	6.92	17.97	29.68	2.72	3.87	5.55
	40	13.10	20.58	21.95	24.52	38.51	41.09	12.01	10.97	10.46
SH150	0	9.01	21.91	33.02	17.62	42.82	64.56	6.26	8.35	10.84
	10	12.46	29.36	42.65	24.41	57.55	83.59	6.81	13.45	19.44
	20	12.22	28.62	41.64	23.26	54.48	79.27	9.35	13.92	19.18
	40	8.77	20.66	30.06	17.22	40.59	59.08	4.28	8.68	12.55
Pooled SEM ^b		2.932	6.411	9.502	5.310	11.721	17.230	1.381	2.063	3.161
Ration effect										
Linear		0.713	0.700	0.821	0.451	0.419	0.506	0.103	0.018	0.027
Quadratic		0.103	0.027	0.015	0.114	0.030	0.016	0.451	0.158	0.045
<i>E. coli</i> effect										
Linear		0.112	0.210	0.270	0.143	0.271	0.361	0.127	0.260	0.409
Quadratic		0.779	0.945	0.975	0.679	0.830	0.904	0.756	0.714	0.534
Cubic		0.762	0.998	0.868	0.740	0.980	0.874	0.866	0.573	0.610
Ration × Dose		0.397	0.496	0.410	0.379	0.469	0.376	0.010	0.129	0.254

^a SH 75, soybean hulls were included at 75 g/kg DM of total mixed ration; SH 150, soybean hulls were included at 150 g/kg DM of total mixed ration.

^b SEM standard error of the mean.

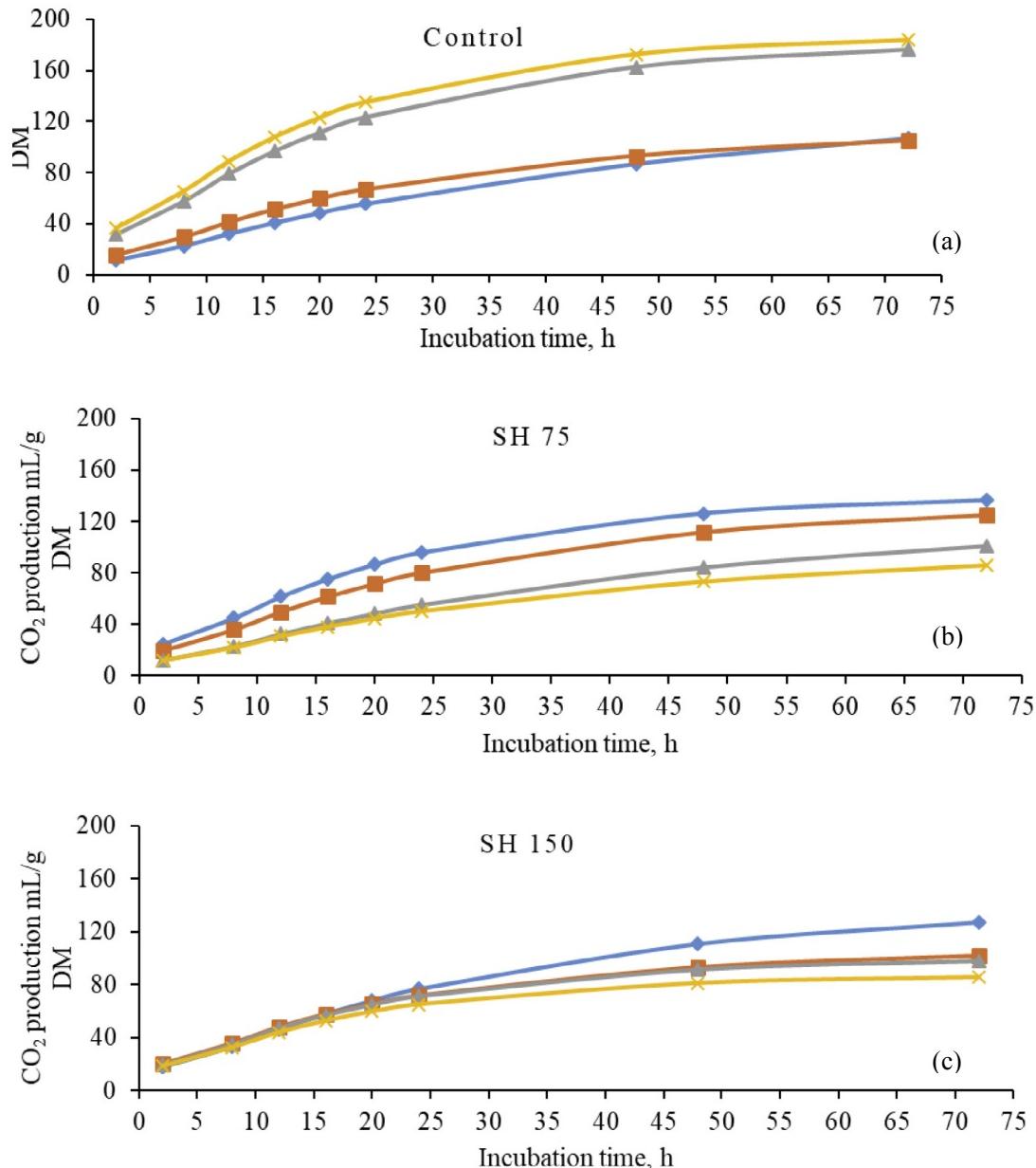


Fig. 4. a-c. *In vitro* CO₂ production (mL/g incubated DM) of three different levels of soybean hulls as influenced by different doses of *E. coli* at 0 (-◆-), 10 (-■-), 20 (-▲-) and 40 (-×-) μL/g DM. Control: corn grain was replaced with soybean hulls at 0 g/kg DM; SH 75: soybean hulls were included at 75 g/kg DM of total mixed ration; SH 150: soybean hulls were included at 150 g/kg DM of total mixed ration.

of CH₄. Elghandour et al. (2016b) reported that replacement of CG with prickly pear cactus flour, SH ration and organic acid salts dose dependent interaction did not influence CH₄ production. Colombatto et al. (2003) observed no influence of enzymes supplementation on CH₄ production while McGinn et al. (2004) reported similar findings in steers fed with barley silage-based diets supplemented with various feed additives. In contrast, Khalif et al. (2016) stated that the supplementation of exogenous enzymes at different doses decreased CH₄ production in ruminant diets. Polvorach et al. (2014) reported decreased in CH₄ production when ruminant animals were fed with *Saccharomyces cerevisiae* fermented cassava chip protein rather than soybean meal. They attributed the reduction in the formation of CH₄ to the ability of *Saccharomyces cerevisiae* to influence H₂ metabolism in the rumen by altering the *in vitro* fermentation. Sahoo and Jena (2014) opined

that organic acid salts have the capacity to reduce methanogenesis via sinking of H₂ during propionate formation. This resulted in high fiber digestion due to stimulated cellulolytic bacteria. Santoso and Hariadi (2009) stated that the slower fermentation of carbohydrates ruminant feedstuffs may be associated with increase *in vitro* CH₄ production. Singh et al. (2012) reported that low protein content of feed diets may contribute to higher CH₄ production because of minimal H₂ and CO₂ production of protein. Moreover, they suggested that protein rich diets have limited potential for methanogenic when compared to carbohydrate diets.

In this present study, replacement of CG with SH has linear effects on asymptotic CO₂ production. No effects or ration and dose interactions were noted at different doses of *E. coli*. The SH 150 had the least asymptotic CO₂ production of 81 mL/g DM at 40 μL/g DM *E. coli* inclusion. This decrease in asymptotic CO₂ production may be

Table 4Proportional *in vitro* CO₂ production as a percent of total gas production of three different levels of soybean hulls (SH) as influenced by different doses of *E. coli* (mg/g DM).

Ration ^a	<i>E. coli</i>	CO ₂ production						Proportional CO ₂ production		
		mL/g incubated DM			mL/g degraded DM					
		8 h	24 h	48 h	8 h	24 h	48 h	8 h	24 h	48 h
Control	0	29.67	71.19	106.41	55.13	132.29	197.74	16.27	22.07	29.14
	10	33.65	72.64	96.19	60.55	130.70	173.06	20.17	27.89	34.70
	20	71.56	151.25	197.18	127.38	268.83	349.95	38.06	45.66	52.62
	40	69.24	140.94	177.53	123.76	252.17	317.92	27.78	37.11	44.42
SH75	0	34.46	76.08	103.10	69.71	153.68	207.94	30.92	36.21	40.80
	10	31.03	72.42	104.75	58.10	135.63	196.26	32.60	37.90	43.89
	20	16.33	42.62	70.31	30.36	79.30	130.94	12.43	17.44	24.59
	40	21.55	47.74	67.60	40.28	89.34	126.69	19.79	25.48	31.40
SH150	0	38.66	89.93	129.54	75.70	176.14	253.83	27.25	33.82	40.95
	10	37.61	69.26	84.12	73.93	135.94	164.90	20.68	31.71	38.34
	20	34.20	70.81	90.26	65.06	134.72	171.73	25.18	32.68	38.88
	40	31.79	62.09	76.01	61.77	120.76	147.95	16.55	28.12	34.14
Pooled SEM ^b		9.502	16.923	20.114	17.031	29.224	33.913	5.404	5.721	5.633
Ration effect										
Linear		0.040	0.011	0.005	0.085	0.022	0.009	0.425	0.697	0.601
Quadratic		0.011	0.011	0.019	0.012	0.010	0.018	0.986	0.387	0.272
E. coli effect										
Linear		0.328	0.540	0.963	0.448	0.742	0.743	0.439	0.833	0.858
Quadratic		0.798	0.870	0.958	0.958	0.908	0.766	0.629	0.643	0.572
Cubic		0.574	0.297	0.149	0.588	0.278	0.121	0.798	0.852	0.877
Ration × Dose		0.051	0.025	0.020	0.044	0.017	0.012	0.035	0.043	0.050

^a SH75, soybean hulls were included at 75 g/kg DM of total mixed ration; SH150, soybean hulls were included at 150 g/kg DM of total mixed ration.^b SEM standard error of the mean.

associated with reduced cell wall content which may lead to reduced microbial activities. The control produced more CO₂ in all the incubation time than any of the SH rations and this result is in agreement with the findings of Kholif et al. (2016), and similar finding was observed by Elghandour et al. (2016a). In their experiment, they reported a decrease in CO₂ production when CG was replaced with SH. In contrast, Elghandour et al. (2016b) reported that replacing CG with prickly pear cactus flour increased CO₂ production. Although both experiments used the same organic acid salts addition, the observed different effects may be due to ration dependent.

E. coli at different levels did not have any effect on the rate of CO₂ and initial delay before CO₂ productions began. This result is similar to the work of Elghandour et al. (2018) who reported that inclusion of prickly pear cactus flour rations and *E. coli* doses had no much influence on the rate of CO₂ and initial delay before CO₂ productions. However, SH ration affected the mL/g incubated DM and mL/g degraded DM of CO₂ production at all incubation time. The control had the highest mL/g incubated DM and mL/g degraded DM CO₂ production when compared with SH rations and *E. coli* doses, thus revealing the ability of SH ration and *E. coli* doses to decrease CO₂ production.

5. Conclusions

Ruminal fermentation is accompanied by large amount of CH₄ and CO₂ productions. Mitigation of these gases will significantly reduce net loss of feed energy and can lead to cleaner environmental production of livestock. This study demonstrated that SH ration and *E. coli* doses had no effect on the CH₄ production and therefore redundant in mitigation of CH₄, however, they have a decreasing effect on asymptotic GP. This study also established that inclusion of SH 150 ration at different *E. coli* doses reduced asymptotic CO₂ production which can be an environmental friendly way of feeding livestock. Further studies are required to evaluate the effectiveness of SH ration along with *E. coli* doses to improve the ruminal fermentation parameters.

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